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Research Article

Isolation and characterization of a novel nitrogen fixer *Beijerinckia fluminensis* strain BAUMS11 from litchi (*Litchi chinensis* L.) rhizosphere

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Abstract
Indiscriminate use of mineral fertilizers has a broad negative impact on soil health. Because of the above, there is an urgent need to search for natural organic alternatives, including using soil microbial resources to replenish soil nutrients for enhanced Agri productivity vis a vis sustainably maintaining soil health. The nitrogen-fixing rhizobacteria (NFR) are such type of bacteria which fix gaseous atmospheric nitrogen in the soil and in nodules of certain plant species in considerable amounts that are readily available for plants' uptake and may be considered as a viable alternative source of mineral nitrogen application. The present study was conducted to isolate the most potent nitrogen-fixing bacteria from the litchi rhizosphere. Hence, Five NFR (NFR1 to NFR5) were isolated from the rhizosphere of litchi orchard of Bihar Agricultural University, Sabour, Bhgalpur, India, based on their ability to fix atmospheric nitrogen in a nitrogen-free mineral salt medium. NFR2 was found to be the most potent in fixing atmospheric nitrogen (11.31 mg N per gram carbon source)among all the isolated rhizobacterial strains. Hence, on the basis of biological nitrogen fixation ability, the isolate NFR2 was subjected to 16S ribosomal RNA (16S rRNA) gene sequencing for molecular characterization. Based on 16S rDNA sequence analysis, NFR2 showed the closest sequence homology with *Beijerinckia fluminensis* and was identified and reported as *Beijerinckia fluminensis* strain BAUMS11, Accession number MN533953. The study noticeably indicated that the *B. fluminensis* strain BAUMS11 was found most efficient in fixing gaseous atmospheric nitrogen and may be used for the manufacturing of nitrogenous biofertilizer, which can fix atmospheric nitrogen to the tune-up to 30 kg N $ha^{-1}yr^{-1}$.

Keywords: *Beijerinckia fluminensis*, Litchi, Nitrogen fixation, Rhizosphere

INTRODUCTION

Nitrogen (N) is one of the most imperative and essential macronutrients, which is needed in large quantities by the crops and plays multiferous roles in plant growth and development, which influences the yield of crops (Singh *et al.,* 2023). This element is considered the main constituent of amino acids, building blocks of plant proteins and enzymes (Bloom, 2015).Plants can absorb nitrogen (N) from a variety of sources, including chemical and organic fertilizers. The most restricting element for plant growth and development is nitrogen,

and the inadequate availability of nitrogen in the rhizosphere of the majority of commercially significant crops has emerged as a key driver of agriculture's sustainable development. Nevertheless, some of the nitrogenous fertilizers with synthetic chemical composition are imported from outside the nation, which could eventually have a detrimental effect on India's agro-based financial system. Furthermore, it has been demonstrated that the widespread application of chemical fertilizers degrades soil structures, reduces soil organic matter (SOM), and intensifies environmental contamination by contaminating underground water. Although the continuous application of mineral fertilizers leads to a decline in SOM content, which is coupled with a decrease in the quality of soil (Pahalvi *et al.,* 2021)

All plants utilize nitrogen in the form of NO_3^-, which significantly increases and enhances the yield and quality by playing a vital role in biochemical and physiological processes in plants. Through the use of the enzyme nitrogenase, which requires 8H+, 8e-, and 16 mg of ATP, atmospheric nitrogen (N_2) is reduced into two molecules of ammonia (NH_3) during the biological nitrogen fixation (BNF) process. Numerous free-living bacteria can release the nitrogenase enzyme, which transforms atmospheric nitrogen gas into a form that plants can utilise. Although, engineering cereal crops that can fix nitrogen, like legumes or associate with nitrogen-fixing microbiomes, could help to avoid the problems caused by the overuse of synthetic nitrogen fertilizer (Guo *et al.,* 2023).

Although nitrogen is abundant in the atmosphere, very few organisms can use it directly; instead, they need fixed nitrogen in the form of ammonium or nitrate, which is why biological nitrogen fixation is a crucial mechanism in supporting plant growth. *Beijerinckia fluminensis* was first reported and described by Dorereiner and Ruschel (1958) as a potent nitrogen fixer strain, though it was found less abundant than *B. indica* in tropical soils from four Brazilian States and in the Congo. The amount of nitrogen fixed by *B. Fluminensis* has been reported to be about 12.9 mg N/g sucrose (Dorereiner and Ruschel, 1958). In another study conducted by Becking (1978) the nitrogen fixing efficiency of *Beijerinckia* in pure cultures was found to be about 16 mg Ng^{-1} glucose consumed. *Beijerinckia* is a slow grower and has been observed to consume only 50% of available glucose even after an incubation period of 20 days (Becking, 1978). Similarly, Sukweenadhi *et al.* (2022) conducted a field experiment on rice crop var. Ciherang at Indonesia observed that treatment with G3 (B. fluminensis) produced the highest average root weight, length, width, root volume and surface area compared to the control and G4c (*Rhizobium pusense*) treatment. Considering the above facts, the present study aimed to isolate and characterise free-living nitrogen fixer from litchi rhizosphere soil.

MATERIALS AND METHODS

Isolation and identification of bacterial strain

Soil sampling and isolation

The litchi orchard at Bihar Agricultural University, Sabour, Bhagalpur, was the sampling site from which soil samples and root hairs were collected. The sampling was done 46 meters above mean sea level and with the GPS coordinates –Longitude 87°2'42"East& latitude 25°15′40″ North.The sampling samples were collected in separate plastic bags, immediately brought to the laboratory, and kept inside the refrigerator at 4°C. After gentle shaking and washing, roots and intact adhering soil were considered rhizosphere samples. Using a nitrogen-free mineral medium, the same was used as the starting material for isolating bacteria (Subba Rao, 1986). One gram portion of the rhizosphere soil samples (roots plus the adhering soil) were ground in a mortar using 10 ml of sterilized distilled water and 10-times serial dilutions (up to 10^8) of the rhizosphere samples were prepared aseptically (Sherpa *et al*., 2021 and Gamit and Amaresan, 2022). Thus, in the present study, five isolates of bacteria (NFR1 to NFR5) were isolated.

Purification of bacterial isolates

Some of the individual colonies of NFR1 to NFR 5 (morphologically different and pigmented) were picked up from plates of different dilutions. These colonies were purified following standard procedures (Smibert and Krieg, 1981). The selected single colonies were streaked repeatedly on the nutrient agar plates until isolated single colonies could be obtained. Further purification of the single colonies was carried out on nutrient agar slants. The single colonies were purified on nutrient agar medium and preserved on slants at 4°C in a refrigerator for further studies.

Strains were cultivated at 30°C in a nitrogen-free mineral medium for upkeep and additional examination. Before use, they were checked for purity by streaking on a nitrogen free mineral medium. After confirmation of their purity, the isolates were used in subsequent experiments.

Screening of isolates for nitrogen fixation

Estimation of nitrogen fixation by Kjeldahl method

The Kjeldahl method was used to estimate the amount of nitrogen fixed by the culture filtrate (Nkonge and Ballance, 1982). The ability of the bacteria isolates to fix nitrogen was tested by Kjeldahl method (Jackson, 1973) in a mineral salts medium free from nitrogen. A loopful of the bacterial inoculum (of each isolate separately) was inoculated into a conical flask with 25 ml of sterilized mineral-based salts (pH-6.5).

One set of conical flasks containing 25 ml of sterilized mineral salt-based medium was kept without culture,

which served as an uninoculated control. All the culture flasks (3 replicates for each isolate), including the controls, were incubated at 37°C for 10 days. All samples were analysed for total nitrogen by Kjeldahl method after 10 days of incubation. After subtracting the quantity of nitrogen in the control samples, the amount of nitrogen fixed by each isolate was reported as mg N fixed g^{-1} of glucose.The sample (25 ml of culture) was taken in a 100 ml digestion flask to which a pinch of digestion mixture containing potassium sulphate, copper sulphate and selenium powder were added in a ratio of 10: 1: 0.1 followed by the addition of10 ml of concentrated sulphuric acid. The contents were thoroughly mixed carefully and slowly swirling in the flask to avoid frothing.They were then digested on an electric digestion rack till a clear solution was obtained. The entire sample was transferred to a distillation flask after washing 3-4 times with distilled water. The contents of the distillation flask were made alkaline with an adequate amount of 40% sodium hydroxide solution. The sample was completely distilled into 10 ml of 3% boric acid solution with bromocresol green and methyl red mixed indicator. Complete ammonia distillation from the sample was ensured by checking with red litmus paper. Then, the boric acid solution was titrated with 0.01 N sulphuric acid. The following formula was used to determine the sample's total nitrogen content:

Total N = (T-B) ×N/ S .....Eq. 1

Where,

T = Sample titre value, ml standard acid

B = Blank titre value, ml standard acid

S = Sample volume in ml

N = Normality of the standard acid

Molecular characterization of bacteria

PCR-amplification and 16S rRNA sequence analysis

The bacterial isolates were multiplied on LBliquid broth for 24 hrs. at 32°C. The cell pellets of respective isolates from 1.5 mL cultures were collected aftercentrifugation for five minutes at 13,000 rpm, followed by washing with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8) and thereafter resuspended in 200 µL of TE ($T_{10}E_1$). The cell lysis was obtained with lysozyme (2 mg/mL; final concentration) and by using SDS (1%) at 37°C for 30 min). The cell lysate was extracted twice with chloroform, followed by two chloroform/isoamyl alcohol (24:1) extractions and RNase treatment.The supernatant of respective samples was treated with 0.1 volume of sodium acetate (3 M, pH 5.2) and 0.5 volumes of isopropanol, followed by incubation at –20°C for 30 min. The precipitated nucleic acids were sedimented by centrifugation for 20 min at 13,000 rpm and the resulting pellet was washed with 70% ethanol before drying under vacuum. Further, 0.8% agarose gel was used to assess the DNA quality and purity under agarose gel electrophoresis, in which a single band of high-molecular-weight DNA was observed. After resuspending the nucleic acid pellets in 100 µL TE, they were utilized as a template (Figure 1A) for the 16S rRNA gene PCR amplification. 5 µL of Taq DNA polymerase (5 U/µL; ThermoFisher), 5 µL of Taq buffer, 5 µL of dNTPs (200 µM), 5 µL (100 ng/µL) of each primer (FP: AGAGTTTGATCCTGGCTCAG; RP: AAGGAGGT GATCCAG CCGCA), 24.5 µL of sterile water, and 1 µL of template DNA were included in each reaction mixture (50 µL). Thirty rounds of temperature cycling (94° C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute) were conducted after the template was denaturated at 94°C for 4 minutes. The incubation period then lasted for 7 minutes at 72°C. A single discrete PCR amplicon band of about 1500 bp was observed when resolved on agarose gel. The PCR products were gel purified by using quick spin (Thermo Fisher) kits and sequenced on BDT v3.1 Cycle sequencing kit on ABI 3130xl Genetic Analyzer (Mullis 1990). The sequences were deposited to GenBank (Accession No. MN533953.1).

Phylogenetic analysis

Based on the Kimura 2-parameter model, the Maximum Likelihood technique was used to infer the evolutionary history (Kimura, 1980). The evolutionary history of the taxa under study is assumed to be represented by the bootstrap consensus tree that was derived from 1000 replicates (Felsenstein, 1985). Branches that are collapsed correspond to partitions that are replicated in less than 50% of bootstrap replicates. Eleven nucleotide sequences were analyzed. First, second, third, and noncoding codon locations were covered. Every position with missing data and gaps was removed. The final dataset contained 1434 locations in total. In MEGA11, evolutionary analyses were carried out (Tamura *et al.,* 2021).

Distance matrix

The number of base substitutions made at each position from different sequences and above the diagonal and the standard error estimate(s) are displayed (Table 1). The study used the Kimura 2-parameter model for the analyses. Eleven nucleotide sequences were analyzed. We provided the codon positions (1st+2nd+3rd+Noncoding).Every position with missing data and gaps was removed. The final dataset contained 1434 locations in total. MEGA 11, evolutionary studies were carried out (Tamura *et al.,* 2021).

Statistical analysis

Statistical analysis was done using Microsoft Excel (Microsoft Corporation, USA), SPSS window version 20.0 140(SPSS Inc., Chicago, USA) and R (3.5).

RESULTS AND DISCUSSION

Nitrogen fixation ability of bacterial isolates

The naturally occurring soil bacteria actively colonize plant roots and, when added to seeds or crops, increase the plants' development and yield (Drahos, 1992). In the present study, five isolates (NFR1 to NFR5) were isolated in a nitrogen-free mineral medium and based on nitrogen fixation (Table 1). TheNFR2 (*B. fluminensis* strain BAUMS11) was fixed significantly more nitrogen fixation by 251.24%, 190.74%, 104.52% and 295.45% when compared with the isolates NFR1, NFR3, NFR4 and NFR5, respectively.However, it was observed that all the isolates except NFR2 were found to be statistically at par with each other. It might be due to the ability of nitrogenase enzyme secretions by the respective isolates. In another study, several bacteria from rhizospheric soils like *Azotobacter* sp., *Azospirillum*sp., *Beijerinckia*sp., *Burkholderia* and *Paenibacillus*sp. have been reported to show significant potential for N-fixing efficacy. The results also conform with the findings of Bhattacharjee *et al*. (2012), who isolated the seventeen strains of nitrogen-fixing bacteria of the genus *Beijerinckia*and identified them based on their morphological and biochemical characteristics.

Description of *Beijerinckia fluminensis* strain BAUMS11

Cells are Gram-negative, regular, non-motile, and slightly curved rods that do not form spores, and they were well grown at pH 6.5.A large number of fimbriae are present around the cell, but no flagella are observed. The bacterial colonies on a mineral medium without nitrogen on agar plate were convex, irregular, creamy in colour, highly viscous and opaque.Following five days of development in a nitrogen-free mineral broth, the medium turned opaque, creamy, and extremely viscous. Molecular nitrogen was fixed under aerobic conditions and requires molybdenum. Similar results were obtained by Krupali and Shelat (2018) who isolated the bacteria and characterized them as *B. Fluminensis* strain AAU K3, *Bacillus safensis* strain AAU K4 and *Pseudomonas aeruginosa* strain AAU K5, which showed the presence of *nif* H gene indicating nitrogen fixing ability.Shwaiman *et al*. (2022) also conducted a study that found that the *B. fluminensis* BFC-33 bearing accession no. MT672580 was isolated from the rhizosphere of potatoes and found to be tolerant to various abiotic stresses, viz. heat, drought, salinity, and heavy metals. Strain BFC-33 demonstrated multifarious plant-growth-promoting (PGP) characteristics, such as P-solubilizationability, and production of indole-3-acetic acid, ACC deaminase, ammonia, siderophore, HCN, EPS and extracellular enzymes.

Table 1. Nitrogen fixation by bacterial isolates

Isolates	mg N fix per g sucrose (Mean of three replications)
NFR1	3.22[b]
NFR2(Beijerinckia fluminensis strain BAUMS11)	11.31[a]
NFR3	3.89[b]
NFR4	5.53[b]
NFR5	2.86[b]

Different letters of small alphabets in the same column indicate significant differences (Duncan's test: $p < 0.05$).

Molecular characterization of selected isolates based on 16S rRNA gene sequence analysis

The isolate "BAUMS11" was further subjected to 16S rRNA gene sequencing and based on sequence analysis, it was identified as a member of *B. fluminensis*. The use of conserved primers allowed amplification of the nearly complete 16 S rRNA gene ORF. PCR products (~1500 bp) obtained from the isolate sequenced directly (Fig. 1). The comparison of the almost complete sequence with the databank showed the highest similarity (98.48 % similarity) with the sequence of *B. fluminensis* (Accession No. NR_116306.1). By aligning the forward and reverse sequence data of 16S rRNA gene sequence using aligner software, the Consensus sequence of 16S rDNA of the isolate *B. fluminensis* "BAUMS11" was generated and compared to global nucleotide sequence database by carrying out BLAST analysis through NCBI GenBank database. The first ten sequences were selected based on maximum identity score and aligned with global 16S rDNA sequences using the multiple alignment software program Clustal W. After that, MEGA 11 software was used to generate a distance matrix and construct a phylogenetic tree . Sample which was labelled as M11 showed the highest

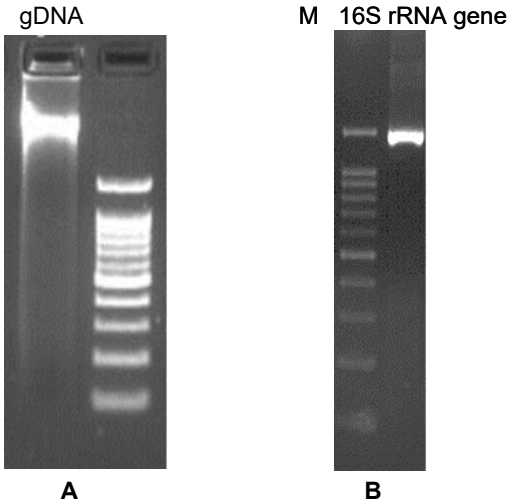

Fig. 1. *Isolation of genomic DNA (gDNA) from isolates (A) and 16S rRNA gene amplification of ~ 1000 bp (B) M DNA Marker/Ladder*

Table 2. Estimates of evolutionary divergence between sequences

M11		**0.002**	**0.002**	**0.002**	**0.003**	**0.004**	**0.002**	**0.004**	**0.004**	**0.004**	**0.004**
NR_116306.1	0.007		0.002	0.001	0.003	0.004	0.002	0.005	0.004	0.005	0.004
NR_115516.1	0.007	0.004		0.001	0.003	0.003	0.003	0.005	0.004	0.005	0.004
NR_041396.1	0.005	0.003	0.001		0.003	0.004	0.003	0.005	0.003	0.005	0.003
NR_074266.1	0.013	0.013	0.017	0.016		0.004	0.002	0.005	0.004	0.004	0.004
NR_115518.1	0.022	0.020	0.016	0.017	0.021		0.004	0.005	0.001	0.005	0.001
NR_116874.1	0.007	0.007	0.011	0.011	0.007	0.027		0.005	0.004	0.005	0.004
NR_044216.1	0.028	0.029	0.029	0.028	0.031	0.032	0.036		0.005	0.005	0.005
NR_118991.1	0.021	0.019	0.017	0.016	0.019	0.001	0.025	0.030		0.005	0.000
NR_159178.1	0.030	0.035	0.034	0.032	0.031	0.036	0.035	0.032	0.035	0.005	0.005
NR_114990.1	0.021	0.019	0.017	0.016	0.019	0.001	0.025	0.030	0.000	0.035	

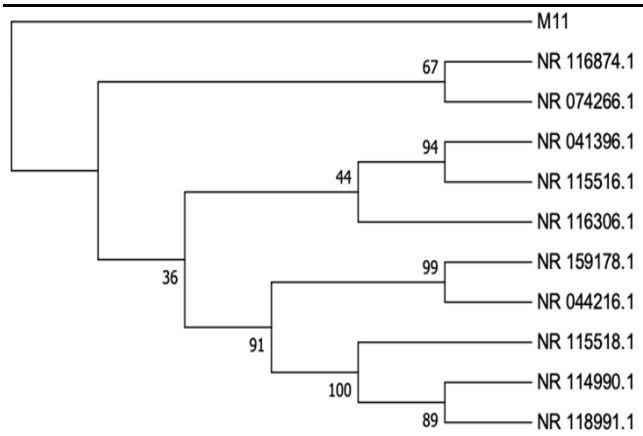

Fig. 2. *Molecular Phylogenetic analysis by Maximum Likelihood method employing MEGA10*

similarity with *B .fluminensis* based on nucleotide homology and phylogenetic analysis (Fig. 2). The nucleotide was submitted online to NCBI and NCBI issued the nucleotide Accession number MN533953 to *B. fluminensis* strain BAMS11. The similar organism *E. cloacae* strain BAB-6019 (GeneBank Accession number KY672863), *Enterobacter cloacae* strain *BAU3* (Accession number MK033472) were isolated by Nagar *et al*. (2017,2018) from maize and rice rhizospheric soils. Similarly, Hind *et al*. (2022) isolated *B. fluminensis* BFC-33 (Accession no. MT672580) from potato rhizosphere and *Bacillus aryabhattai* strain BAUMS8 (Accession number MN533952) (Singh *et al*. 2022).

Isolate (M11) showed high similarity with *B. fluminensis* based on nucleotide homology and phylogenetic analysis [Accession No.: NR_116306.1: *B. fluminensis;* NR_115516.1: Agrobacterium tumefaciens; NR_041396.1: *Agrobacterium tumefaciens;* NR_074266.1: *Agrobacterium fabrum*; NR_115518.1: *Agrobacterium rubi;* NR_116874.1: *Rhizobium pusense*; NR_044216.1: *Rhizobium selenitireducens;* NR_118991.1: *Agrobacterium rubi;* NR_159178.1: *Ciceri bacterthiooxidans;* NR_114990.1: *Agrobacterium rubi]*

Conclusion

A total of 5 isolates were isolated from five litchi rhizosphere soil samples from the litchi orchards of Bihar Agricultural University, Sabour, Bhagalpur. NFR2 was the most potent in fixing atmospheric nitrogen out of the five isolates. NFR2 was identified as *Beijerinckia fluminensis* strain BAMS11(Genebank Accession Number MN533953) by 16s rDNA sequencing. The isolated bacterial strain seemed to be highly potent in fixing atmospheric nitrogen. Thus, this strain can be utilized to prepare biofertilizers and other possibilities for insoluble phosphorus fixation and plant growth hormone secretions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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